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Remarks

In the present continuation application, claims 1-20 have been cancelled without prejudice.

New Claims 21-36 have been added. No new matter has been added as these Claims have support in the specification as discussed below.

Upon entry of the amendment, Claims 21-36 will be pending.

Support for new Claims 21-36 can be found at page 1, lines 16-19; page 17, lines 14-18; page 19, lines 14-16 and lines 26-27; page 20, lines 8-9 and page 21, lines 8-9 of the specification.

Invention Synopsis

The present invention relates to oral compositions suitable for loosening or removing plaque and reducing dental nerve and/or dentin sensitivity with a non-menthol flavoring, comprising an orally-acceptable, soluble potassium salt; a sodium (C8-C24) alkyl sulfate; an orally-acceptable polar surfactant; and an orally-acceptable aqueous vehicle wherein the potassium salt is dissolved in the composition and wherein the molar ratio of the polar surfactant to the sodium alkyl sulfate is greater than or equal to about 1:1 such that when the potassium salt, alkyl sulfate and polar surfactant are dissolved in aqueous vehicle, the resultant composition is clear.

Rejection Under 35 USC §§ 102 and 103

In parent application, the claims were rejected under 35 USC 102(b) as anticipated or , in the alternative under 35 USC 103 as being unpatenable over US Patent 6,193,958 to Edwards et al. (Edwards); U.S. Patents 5,827,505; 6,004,538 and 6,294,154 to Hughes et al. (collectedly referred to as "Hughes"); US Patents 5,681,549 and 5,686,063 to McLaughlin et al. (collectively referred to as "McLaughlin") and U.S. Patent 5188822 (2/93) to Viccaro et. al. ("Viccaro").

Applicants respectfully traverse this rejection.

Edwards relates to oral compositions comprising, inter alia, an aminoalkylsilicone and a silicone surfactant. Edwards further mentions the optional use of surfactants such as alkyl sulphates or sarcosinates and desensitizing agents such as potassium nitrate or potassium citrate. Nowhere, however, does Edwards address or even mention the compatibility issues associated with combining potassium salts with alkyl sulphate surfactants.[1] In contrast, the compositions of the present invention incorporate orally-acceptable polar surfactants to specifically address compatibility concerns when soluble potassium salts and sodium (C8-C24) alkyl sulfates are both required in the composition. Therefore, since Edwards nowhere teaches or suggests incorporating the select polar surfactants of the present invention to reduce the compatibility issues associated with combining alkyl sulphate surfactants with potassium salts, the compositions of the present invention are not anticipated by nor would they have been obvious over this reference.

[1] "While sodium dodecyl sulfate (which is synonymous with SLS) solubility is relatively high (1g/10ml, i.e. 0.3M) on addition of KCL it falls almost to zero..." Carraro, U., *Effective recovery by KCl precipitation of highly diluted muscle proteins solubilized with sodium dodecyl sulfate*, Electrophoresis 1005, 1010 (1991) (copy attached).

Hughes relates to dimethicone copolyol containing oral compositions. Hughes further mentions the optional use of surfactants such as alkyl sulphates or sarcosinates and desensitizing agents such as potassium nitrate or potassium citrate. Like Edwards, however, Hughes nowhere mentions nor addresses the compatibility issues associated with combining potassium salts with alkyl sulphate surfactants[2]. In contrast, the compositions of the present invention incorporate orally-acceptable polar surfactants to specifically address compatibility concerns when soluble potassium salts and sodium (C8-C24) alkyl sulfates are both required in the composition. Therefore, since Hughes nowhere teaches or suggests incorporating the select polar surfactants of the present invention to reduce the compatibility issues associated with combining alkyl sulphate surfactants with potassium salts, the compositions of the present invention are not anticipated by nor would they have been obvious over this reference.

McLaughlin relates to mouthrinse compositions containing quaternary ammonium compounds and polyhydric alcohols. And, Viccaro relates to oral, oil-in-water emulsion compositions where the aqueous phase of the emulsion contains an emulsifier and the oil phase includes a noncyclic, hydrophobic aminoalkyl silicone and a lipophilic compound, which is soluble in the aminoalkyl silicone. Importantly, both McLaughlin and Viccaro are silent regarding the use of sodium lauryl sulfate ("SLS"). In contrast, the present invention specifically targets compositions requiring the use of compounds such as SLS. In particular, the present invention aims at improving the stability of such SLS containing compositions. Therefore, since neither McLauglin nor Viccaro teach or suggest using SLS, the compositions of the present invention are not anticipated by nor would they have been obvious over this reference.

In light of the remarks made herein, it is respectfully submitted that the Examiner's rejection under 35 USC §102 or, in the alternative, §103 in the parent has been overcome. Applicants respectfully submit that they have distinguished the cited art sufficiently to avoid the Examiner's rejection. Accordingly, reconsideration and allowance of Claims 21-36 are earnestly solicited.

Respectfully submitted,

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By _____

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January 9, 2004

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[2] See id. at 1010.

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Effective recovery by KCl precipitation of highly diluted muscle proteins solubilized with sodium dodecyl sulfate

A new, improved method is described, which takes advantage of the low solubility of potassium dodecyl sulfate (KDS), to concentrate sodium dodecyl sulfate-solubilized proteins from nanomolar solutions by KDS precipitation. The method was applied to proteins differing in M_r and p*I*. The effect of varying KCl, and the pH and/or concentration of the buffer on KDS-protein precipitation was studied. These parameters may be chosen to allow selective repartition of specific proteins in the pellet or supernatant. After precipitation, the hundred- or thousand-fold concentrated solutes are easily resuspended in small volumes of any required medium. Though initially experimented to recover muscle proteins from effluents of electroendosmotic preparative gel electrophoresis, the method proved to be of general interest as a powerful tool to recover proteins from highly diluted solutions.

1 Introduction

A common problem in protein chemistry is the concentration of diluted solutes after chromatography or preparative electrophoresis, or from culture media following fermentation. Since many of the older techniques, *e.g.*, ammonium sulfate precipitation, dialysis and lyophilization, possess the considerable drawbacks of low recovery and degradation of labile proteins, great efforts have been applied to develop rapid, efficient, and milder methods. Membranes for ultrafiltration and dialysis allow the concentration and desalting of proteins with good yields and little denaturation, but the handling of diluted proteins, of less than 1 µg/mL, remains a difficult problem [1]. This paper describes a method for the recovery of microgram quantities of proteins in large volumes of highly dilute solutions and its application to muscle proteins, which can be effectively precipitated from solutions of less than 0.1 µg/mL.

Although contractile proteins are the most abundant components of skeletal muscle, some of their isoforms are present only in trace amounts [2–7]. Myosin is a hexapolypeptide consisting of two heavy chains (MHC, 200 kDa) and four light chains (MLC around 20 kDa), which are usually analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional PAGE [8–13]. SDS-PAGE in 6% gels separates the MHC isoforms of adult muscle into four distinct bands: MHC2S, MHC2A, MHC2B, and MHC1 [14–21]. Additional MHC are present during myogenesis of developing and regenerating muscles [2–7]. By electroendosmotic preparative gel electrophoresis (EPGE) it is possible to collect single MHC isoforms in almost pure molecular form from complex mixtures of crude myosins or from tissue homogenates [22]. However, because of the low muscle content of MHC2S, peptide mapping of the purified protein was difficult because of its low concentration in EPGE fractions. The standard methods of protein concentration proved only partially successful [23, 24]. A new approach based on the precipitation of SDS-solubilized protein by the addition of KCl solves the problem. Indeed, the method takes advantage of the low solubility of the potassium salt of dodecyl sulfuric acid (KDS). This simple and efficient technique was brought to our mind by the well-known fact that soaking gel slabs in concentrated KCl solutions visualizes protein bands [25]. Protein band visualization by KCl is a useful tool in gel electrophoresis, but the principle may have greater potential in the field of molecular biology. We have now identified the factors necessary for the successful recovery of SDS-solubilized proteins by KCl addition (KDS-protein precipitation). After concluding these experiments we became aware of the fact that the KDS-protein precipitation had previously been used to separate total viral proteins from highly concentrated solutions (more than 1 mg/mL) of the double-stranded genomic RNAs of the bluetongue virus [26]. We have extended the method to the case of highly diluted solutions. In particular we demonstrate that it is possible to collect, by KCl precipitation, µg amounts of MHC from EPGE effluents. Furthermore, we show that selective repartition of peculiar proteins from a mixture as complex as a tissue homogenate is also feasible by an experimental variation of the pH and buffer before the addition of KCl.

2 Materials and methods

2.1 Myosin extraction

Myosin was extracted essentially according to Carraro *et al.* [11]. All procedures were performed at 2–4°C. Muscle tissue was homogenized (Polytron PT 10 OD), using 50 mM KCl containing 10 mM [ethylene-bis(oxyethylenenitrilo)]-tetraacetic acid (EGTA), 200 µg/L pepstatin, 1 mM benzamidine (Sol A), to reduce the effects of endogenous proteases. After centrifugation at 650 × *g* for 10 min, the pellet was resuspended and centrifuged in Sol A three or more times until the supernatant became clear. The myosin was then extracted with 0.3 M KCl, 10 mM Mg acetate, 0.15 M

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Abbreviations: EDL, extensor digitorum longus; EGTA, ethylenebis-(oxyethylenenitrilo)tetraacetic acid; EPGE, electroendosmotic preparative gel electrophoresis; KDS, potassium dodecyl sulfate; KDS-proteins, potassium dodecyl sulfate-protein complexes; MHC, myosin heavy chains; MLC, myosin light chains; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris, Tris (hydroxymethyl) aminomethane

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KH_2PO_4-K_2HPO_4, pH 6.5, supplemented just before use with 3 g/L ATP (Sol B). Appr ximately 25 mL of Sol B was used per gram of fr sh muscle. After 30 min, samples were centrifuged at 20 000 × *g* for 20 min and then at 150 000 × *g* for 2 h. The supernatant was dialyz d for 4–12 h in 1 mM Tris-HCl, pH 7.2, 10 mM KCl, 0.1 mM dithiothreitol, and 5 mM EGTA. Myosin was collected by centrifugation at low speed, dissolved in 0.5 M NaCl, 50% v/v glycerol, and stored at –20°C. When myosin was needed it was precipitated by dilution, at a low ionic strength, with ten volumes of cold water and centrifugation at 650 × *g* for 10 min.

2.2 SDS-PAGE

Analytical SDS-PAGE of MHC was performed on 6% polyacrylamide (29.2:0.8 of acrylamide/*N,N'*-methylenebisacrylamide) slabs, 0.75 × 130 × 130 mm. Slabs were prepared according to Laemmli [27], but 37.5% v/v glycerol was present in the separating and in the stacking gel [28]. The electrophoretic buffer was 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3, (Sol C). Overnight separation of MHC was achieved with the constant-current mode at about 6 mA per slab, which corresponds to an initial voltage of 40 V between the electrodes. Usually gel electrophoresis was started in the late afternoon and the dye front ran out of the slab overnight; the run was terminated after 16–18 h of electrophoresis, at which time the voltage had risen to 180–200 V. SDS-PAGE in 12.5 or 15% gels (29.2:0.8 of acrylamide/*N,N'*-methylenebisacrylamide) were used to analyze albumin, myosin light chains, or total proteins of the muscle homogenate (see legends of the figures). When the separated bands contained more than 0.2 µg of protein, the slabs were stained with 0.1% Coomassie Brilliant Blue R-250 in 5% acetic acid and 40% ethanol, and destained with 7% acetic acid, 40% methanol. If the bands contained less than 0.1 µg of protein the slabs were silver-stained [29]. Densitometric scanning of gel patterns was performed using a GS 300 Transmission/Reflectance Scanning Densitometer (Hoefer Scientific Instruments, San Francisco) c nnected to a MacIntosh SE (Apple Computer). Muscle h mogenate, myosin and myosin heavy chains, prepared and stored as described by Carraro *et al.* [11], were analyzed by SDS-PAGE either before or after EPGE purification [22]. Commercial albumin was also used. Muscle homogenate or purified proteins, dissolved in 10% w/v glycerol, 2.3% SDS, 5% v/v 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8 (Sol D) at a final concentration of 1 mg/mL, were incubated at 0°C for 30 min and then diluted to 50 µg/mL of different solutions to study the effects of pH and different buffer concentrations.

2.3 Precipitation of SDS-solubilized proteins

In the control experiments, aliquots of proteins dissolved in 10% w/v (1.08 M) glycerol, 2.3% SDS, 5% v/v 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8 (Sol D), at 0°C for 30 min were diluted to 50 µg/mL with one of the following solvents: (i) H_2O; (ii) Sol C; (iii) 25 mM Tris, 192 mM glycine, pH 8.3, (Sol C, but without SDS = Sol E); 125 mM Tris, 960 mM glycine, pH 8.3 (5 × Sol C, but without SDS = Sol F). Diluted protein solutions were divided into aliquots of 5 mL, to which 0.5 mL of KCl at different c ncentrations were added (see legends of the figures). All procedures were performed at 0°C if not otherwise stated. Conical glass test tubes were used to improv the packing of the pellets. Samples were centrifuged at 650 × *g* f r 15 min. The supernatants wer collected and the pellets immediately resuspended by adding 100 µL of Sol G (10% glycerol, 5% 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, identical to Sol D but without SDS). SDS-PAGE f aliquots of th supernatant (usually 20 µL from 5.5 mL) and of the resuspended pellets (usually 20 µL from 100 µL) was either performed immediately or after storage at –20°C until use.

2.4 KDS-protein precipitation: Standard procedure

After studying the factors influencing precipitation and recovery of proteins, the following standard procedure of KDS-protein precipitation was defined to obtain an effective recovery of highly diluted proteins: (i) Native proteins: To a dilute solution of proteins an equal volume of 0.2% SDS, 62.5 mM Tris-HCl, pH 6.8, is added, followed by either boiling for 3 min or incubating for 30 min on ice. (ii) Proteins already solubilized with SDS: Add 25 mM Tris, 192 mM glycine, pH 8.3 (Sol E) to dilute SDS to a final concentration of 0.1–0.2%. (iii) Add ice-cold 100% trichloroacetic acid (TCA) to lower the pH to less than 3.0 (usually to a final concentration of 10% TCA). (iv) Keeping the acidified solution on ice, add 2 M KCl to a final concentration of 180 mM. (v) Centrifuge in conical test tubes at 650 × *g* for 15 min at 2–4°C. (vi) Remove the supernatant. (vii) If SDS-PAGE has to be performed, KDS-protein pellets are resuspended with 10% glycerol, 62.5 mM Tris-HCl, pH 6.8. Aliquots of the milky KDS-protein suspension are loaded into the PAGE wells. During electrophoresis the dodecyl sulfate ions replace protein-bound KDS, solubilizing the pro-

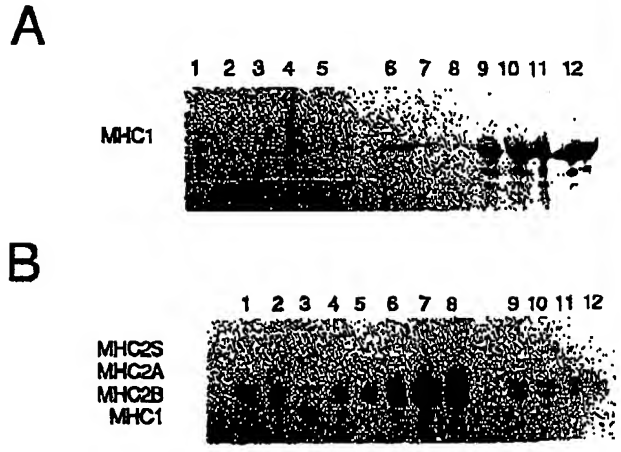

Figure 1. TCA or KCl precipitation of 0.1% SDS-proteins. SDS-PAGE in a 6% gel, followed by Coomassie Blue staining. TCA- or KCl-pellets from 5 mL aliquots of EPGE-purified MHC1 were resuspended in 100 µL of Sol G. (A) Lane (1) EPGE-purified MHC1 from soleus myosin, 1 µg; (2)–(3), 10–20 µL of supernatant after TCA addition (10% final concentration) and high speed centrifugation; (4)–(5) 20–30 µL of solubilized TCA precipitates; (6) EPGE-purified MHC1 from soleus myosin, 50 ng; (7)–(8), 10–20 µL of supernatant after KCl addition (90 mM final concentration) and low speed centrifugation; (9)–(12); 10, 20, 30, and 40 µL of solubilized KCl precipitates. (B) Lane (1) diaphragm myosin, 1 µg; (2) EDL myosin, 1 µg; (3) soleus myosin, 1 mg; (4) EDL myosin diluted with Sol C to 50 µg/mL (sample before KCl addition); MHC precipitated after addition of KCl at a final concentration of KCl: (5) 3.6 mM; (6) 18 mM; (7) 90 mM; (8) 180 mM; (9)–(12) supernatants of samples (5)–(8).

teins. Alternatively KDS-protein pellets can be solubilized with Sol D. Proteins solubilized by an excess of SDS can be renaturated by efficient methods of detergent removal and protein ref lding before storage or further analyses [25, 30–33].

3 Results

Precipitation, on the basis of solubility in an aqueous solution of salts and organic solvents, is one of the better established methods for protein recovery, but often the low concentration of the required solute does not permit this approach [34]. Figure 1A shows that SDS-solubilized proteins, which do not precipitate satisfactorily after acidification with TCA (final concentration, 10% TCA; 30 min on ice) and high speed centrifugation (lanes 2–5), are effectively precipitated by the addition of KCl (final concentrati n 90 mM KCl, 10 min on ice) and low speed centrifugation (lanes 7–12). In order to optimize KDS-protein precipitation we used different amounts of KCl with a constant SDS concentration (0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3). Figure 1B shows the effects on precipitation of increasing amounts of KCl (from 3.6 to 180 mM final concentration). Densitometric scanning of gel patterns confirms the visual impresion that 90–180 mM KCl effectively preci-

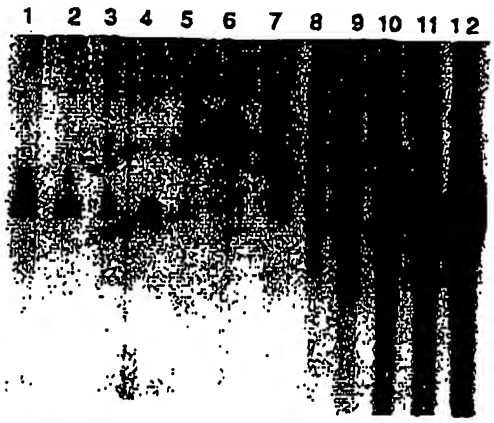

Figure 2. Effect of protein concentration on KDS-protein precipitation. Aliquots of 5 mL of EDL myosin in 0.5% glycerol, 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3 (at the protein concentration stated below) were brought to 180 mM KCl. Pellets were resuspended in 100 µL of Sol E, and 40 µL were loaded per well. SDS-PAGE in a 6% gel, followed by silver staining. (1)–(6) EDL myosin 200, 100, 50, 25, 10, and 5 ng, respectively; (7)–(12) KDS-protein precipitates from EDL myosin solutions at 0.1, 0.5, 1, 5, 10 and 20 µg/mL, respectively. Note that effective recovery is obtained even with the most diluted solution (500 ng of myosin in 5 mL), corresponding to a concentration of a 0.5 mM solution by a factor of 50 in one step.

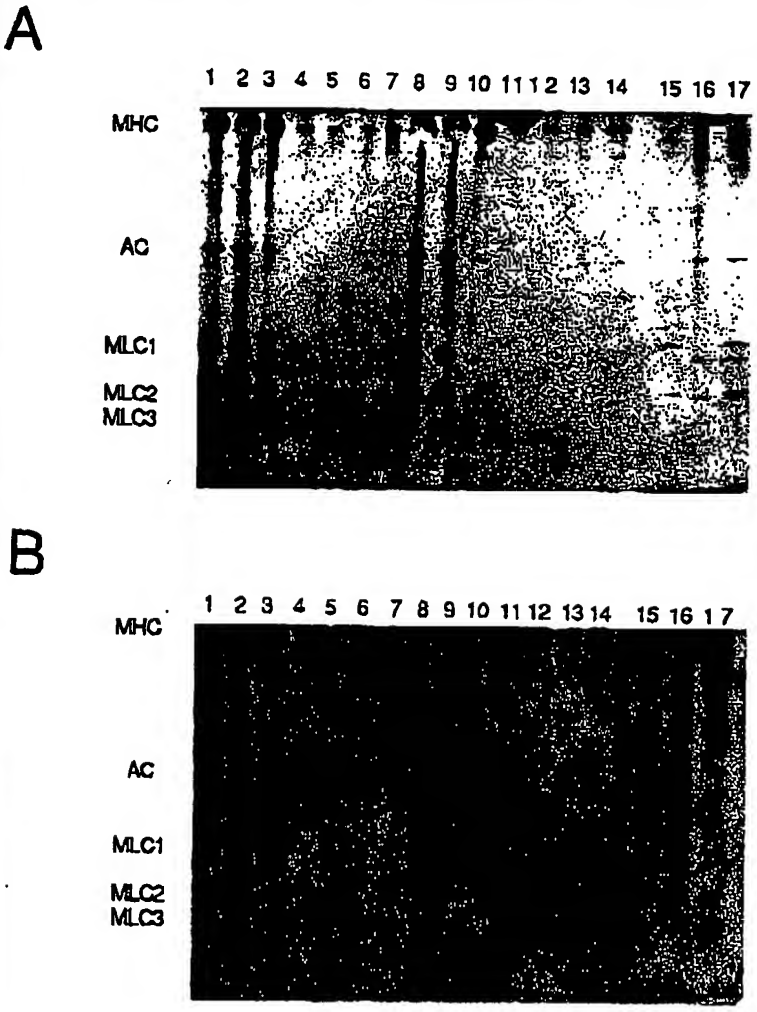

Figure 3. Effects of pH buffer composition and KCl concentration on KDS-protein precipitation of myosin and EPGE-purified MHC. SDS-PAGE, in a 12.5% gel, followed by silver staining. (A) Lanes (1)–(3) KDS-protein pellets, and (4)–(6) supernatants from EDL myosin in 0.5% glycerol, 0.1% SDS, 3 mM Tris-HCl, pH 6.8, brought to 180, 90, and 36 mM KCl, respectively; (7) EDL myosin, 1 µg (sample before KCl addition); (8)–(10) KDS-protein pellets, and (11)–(13) supernatants from EDL myosin in 0.5% glycerol, 0.1% SDS, 125 mM Tris, 960 mM glycine, pH 8.3, brought to 180, 90, and 36 mM KCl, respectively; (14) EDL myosin, 1 µg (sample before KCl addition); (15) soleus myosin, 1 µ; (16) EDL myosin, 1 µg; (17) soleus myosin, 1 µg, and EDL myosin, 0.5 µg. (B) Lanes (1)–(3) KDS-protein pellets, and (4)–(6) supernatants from EDL myosin in 0.5% glycerol, 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3, brought to 180, 90, and 36 mM KCl, respectively; (7) EDL myosin, 1 µg; (8)–(10) KDS-protein pellets, and (11)–(13) supernatants from EPGE-purified MHC brought to 180, 90, and 36 mM KCl, respectively; (14) EPGE-purified MHC (sample before KCl addition); (15) soleus myosin, 1 µg; (16) EDL myosin, 1 µ; (17) soleus myosin, 1 µg, and EDL myosin, 0.5 µg.

Table 1. Effect of KCl concentration on KDS-protein precipitation of MHC[a]

KCl Final concentration	Pellet OD (arbitrary units)	Supernatant
0	–	5,840
4	1,980	6,170
18	24,740	5,080
90	57,500	3,100
180	66,900	50

a) Effect of increasing amount of KCl in presence of 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3. Data from Fig. 1B.

pitates SDS solubilized proteins (Table 1). Figure 2 impressively demonstrates the sensitivity of the method. MHC at 100 ng/mL are effectively precipitated from 5 mL of 0.5% glycerol, 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3, by the addition of 0.5 mL of 2 M KCl (lane 7). Note that the band thickness in lane 7 is comparable with that of the 100 ng marker of MHC (lane 2). Lanes 8–12 show increasing amounts of MHC, thus confirming that myosin heavy chains are effectively precipitated by 180 mM of KCl from 5 mL aliquots of myosin solutions at 0.5, 1, 5, 10, and 20 μg/mL.

The results of different KCl concentrations on precipitation of myosin from solutions differing in pH and concentration of buffer chemicals are reported in Fig. 3. Myosin solubilized in 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, at 1 mg/mL and then diluted to 50 μg/mL with cold water (that is, at a final concentration of 0.5% glycerol, 0.1% SDS, 3 mM Tris-HCl, pH 6.8) or 25 mM Tris, 192 mM glycine, pH 8.3, is effectively precipitated by 180 mM KCl (Fig. 3A, 1 and B, 1 and 8). On the other hand, a higher concentration of an alkaline buffer (125 mM Tris, 960 mM glycine, pH 8.3) interferes with KCl precipitation of SDS-solubilized myosin (Fig. 3 A, 8–13). The decreased precipitability of myosin subunits in a highly concentrated alkaline buffer is even more evident at lower KCl concentrations (compare Fig. 3 A, 9 and 10 with B 2 and 3).

Figure 4 A confirms that an efficient KDS-protein precipitation occurs at a neutral or mildly acidic pH (compare lanes 2 and 3 with lanes 6 and 7 for albumin, or lanes 9 and 10 with 13 and 14 for myosin, pH 8.3 and pH 4.0, respectively). The ability of KCl to precipitate these proteins is always greater than that at TCA alone (compare lanes 6 and 7 in Fig. 4 A with lanes 16 and 17). Figure 4 B shows that albumin is effectively precipitated at pH 1 when KCl (final concentration 180 mM) is added after acidification of the protein solution with TCA (Table 2). The well-known principle of decreased

Table 2. KDS-protein precipitation at 180 mM KCl: Effect of pH on recovery of SDS-solubilized albumin, MLC and MHC[a]

pH	KCl	Protein precipitation		
		Albumin	MLC	MHC
11.0	180 mM	+	+	+++
10.0	180 mM	+	+	+++
8.3	180 mM	++	++	++++
7.0	180 mM	+++	+++	++++
4.0	180 mM	++++	++++	++++
1.0	180 mM	+++++	+++++	+++++
1.0	No KCl	++	+	+

a) Aliquots of either albumin or myosin were diluted to 0.1% SDS, 25 mM Tris, 192 mM glycine, buffered to pH indicated. 2 M KCl was then added to a final concentration of 180 mM. Data from Fig. 1 A, 4 A, B and 5. Protein precipitation: poor +, moderate ++, good +++, very good ++++, excellent +++++, was estimated by visual inspection of protein bands in SDS-PAGE analyses of KDS-protein pellets and supernatants.

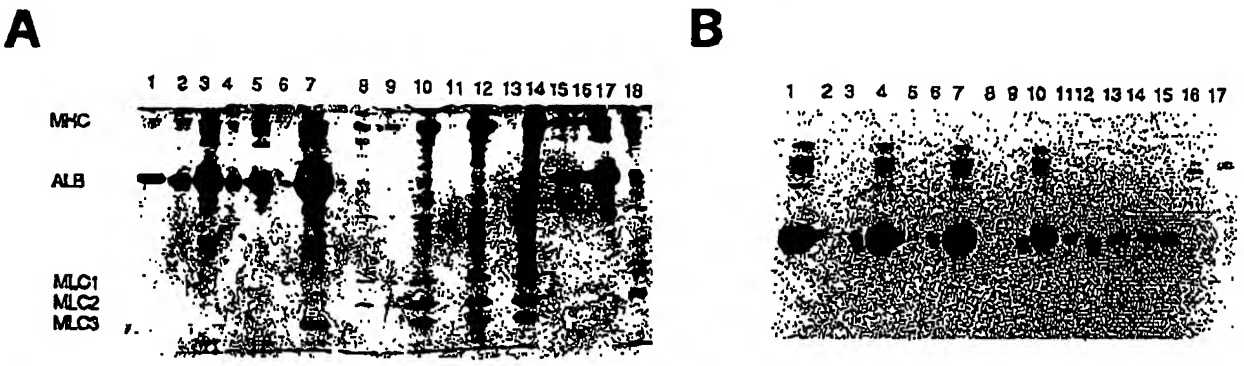

Figure 4. Effects of pH on precipitation by 180 mM KCl (final concentration) of SDS-solubilized albumin and myosin. SDS-PAGE in a 12.5% gel, followed by silver staining. Lane (1) albumin, 50 μg/mL, in 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3 (sample before KCl addition), 1 μg; (2) supernatant and (3) KDS-albumin precipitate; (4) supernatant and (5) KDS-albumin precipitated from sample (1) buffered with TCA to pH 7.0 before KCl addition; (6) supernatant and (7) KDS-albumin precipitated from sample (1) buffered with TCA to pH 4.0 before KCl addition; (8) myosin, 50 μg/mL, in 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3 (sample before KCl addition), 1 μg; (9) supernatant and (10) KDS-myosin precipitated after KCl addition to sample (8); (11) supernatant and (12) KDS-myosin precipitated from sample (8), buffered with TCA to pH 7.0 before KCl addition; (13) supernatant and (14) KDS-myosin precipitated from sample (8), buffered with TCA to pH 4.0 before KCl addition; (15) albumin, 50 μg/mL, in 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3 (sample before TCA addition), 1 μg; (16) supernatant and (17) albumin precipitated after TCA (but not KCl) addition and high speed centrifugation (150 000 × g per 120 min); (18) myosin marker, 1 μg. (B) Coomassie Blue staining. Albumin, 50 μg/mL, in 0.1% SDS, 25 mM Tris, 192 mM glycine, buffered with TCA to pH 1.0 before KCl addition. Lane (1) KDS-albumin precipitate, (2) supernatant, (3) albumin before KCl addition, 1 μg. Albumin, 50 μg/mL, in 0.1% SDS, 25 mM Tris, 192 mM glycine buffered with TCA to pH 4.0 before KCl addition. (4) KDS-albumin precipitate, (5) supernatant, (6) albumin before KCl addition, 1 μg. Albumin, 50 μg/mL, in 0.1% SDS, 25 mM Tris, 192 mM glycine, buffered with TCA to pH 7.0 before KCl addition. (7) KDS-albumin precipitate. (8) supernatant. (9) albumin before KCl addition. 1 μg. Albumin, 50 μg/mL, in 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3, before KCl addition. (10) KDS-albumin precipitate, (11) supernatant, (12) albumin before KCl addition, 1 μg Albumin, 50 μg/mL, in 0.1% SDS, 25 mM Tris, 192 mM glycine, buffered with NaOH to pH 10.0 before KCl addition. (13) KDS-albumin precipitate, (14) supernatant, (15) albumin before KCl addition, 1 μg; (16) rat soleus MHC markers; (17) rat EDL MHC markers. Note that at neutral and basic pH albumin remains partially in the supernatants.

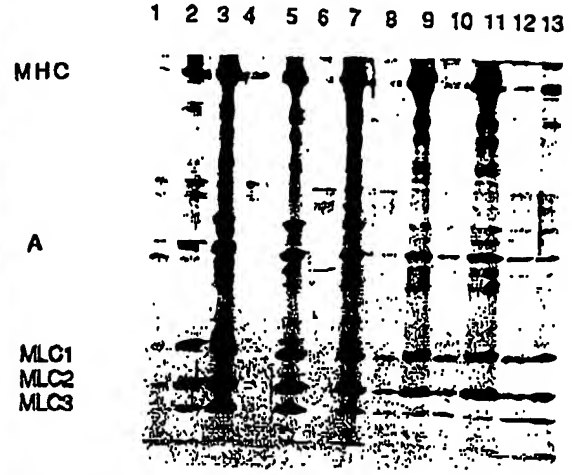

Figure 5. Effects of pH on differential precipitation by 180 mM KCl (final concentration) of SDS-solubilized myosin subunits. SDS-PAGE in a 12.5% gel, followed by silver staining. Rat EDL myosin, 50 µg/mL, in 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3, treated with TCA to pH 1.0 (but not KCl) and high speed centrifugation. (1) TCA precipitate and (2) supernatant; TCA to pH 1.0, and KCl, and low speed centrifugation. (3) KDS-precipitate, (4) supernatant; TCA to pH 4.0 and KCl: (5) KDS-precipitate, (6) supernatant; TCA to pH 7.0 and KCl: (7) KDS-precipitate, (8) supernatant; without TCA (pH 8.3), but with KCl: (9) KDS-precipitate, (10) supernatant; NaOH to pH 10.0 and KCl: (11) KDS-precipitate, (12) supernatant; (13) rat EDL myosin, 50 µg/mL, in 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3 (sample before TCA and KCl addition). Note that lanes (10) and (12) show that at alkaline pH the bulk of MLC, but not of MHC, remains in the supernatants.

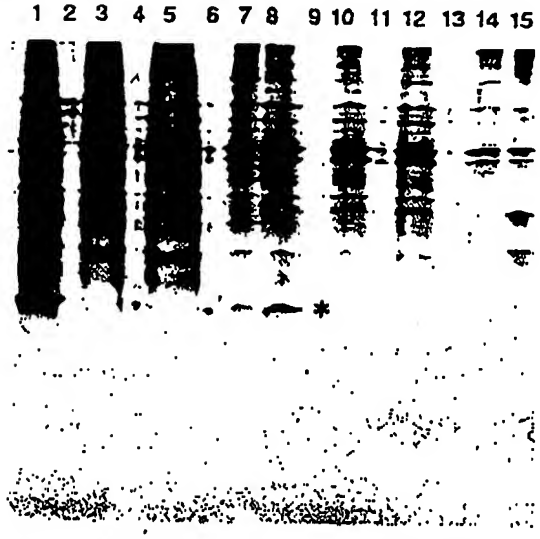

Figure 6. Effects of pH on precipitation by 180 mM KCl (final concentration) of total muscle proteins. SDS-PAGE in a 15% gel, followed by silver staining. Proteins of muscle homogenate, 40 µg/mL, in 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3, treated with TCA to pH 4.0 and KCl: Lane (1) KDS-precipitate and (2) supernatant; without TCA (pH 8.3), but with KCl: (3) KDS-precipitate and (4) supernatant; NaOH to pH 10.0 and KCl: (5) KDS precipitate and (6) supernatant. (7) Proteins of muscle homogenate, 40 µg/mL, in 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3 (sample before treatments). Proteins of muscle homogenate, 10 µg/mL, in 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3, treated with TCA to pH 4.0 and KCl: (8) KDS-precipitate and (9) supernatant; without TCA (pH 8.3), but with KCl: (10) KDS-precipitate and (11) supernatant; NaOH to pH 10.0 and KCl: (12) KDS-precipitate and (13) supernatant. (14) Proteins of muscle homogenate, 10 µg/mL, in 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3 (sample before treatments). (15) Myosin marker.

solubility of SDS at an acidic pH also applies to KDS-protein precipitation. However, Fig. 5 shows that a selective precipitation of some myosin subunits is achieved when KCl is added at an alkaline pH. After addition of KCl to an SDS-myosin solution buffered at pH 10, a large proportion of MLC is contained in the supernatant (lane 12), while MHC precipitates out and is therefore present in lane 11. It is worth noting that TCA precipitation alone, from this very diluted SDS-myosin solution, is unsatisfactory (lanes 1 and 2).

Figure 6 shows the results of precipitating proteins of total muscle homogenate after SDS solubilization, dilution to 40 or 10 µg/mL at a different pH (4.0, 8.3, and 10) and KCl addition (180 mM final concentration). Total proteins are more effectively precipitated at an acidic pH, but they are generally also precipitated at an alkaline pH. A low-molecular-mass band, indicated by an asterisk in Fig. 6, appears in the resolubilized precipitate after acid KCl precipitation (Fig. 6, 1), whereas it remains in the supernatants when KCl addition is performed at a neutral or an alkaline pH (Fig. 6, 4 and 6, respectively). It is, therefore, a more general phenomenon that, at an alkaline pH, KDS-binding to proteins varies, allowing a selective repartition of specific proteins in pellets or supernatants.

4 Discussion

Dodecyl sulfate salts have a low solubility at a low temperature and an acid pH and the potassium salt is almost insoluble. This fact has been used to reveal protein bands in gel slabs [25], to decrease the concentration of SDS in solution [33] and to separate, in a highly concentrated solution, total nucleic acid from viral proteins [26]. We have extended the method to the case of highly diluted solutions, showing that selective repartition of specific proteins from a mixture as complex as a tissue homogenate is also feasible by varying the pH and buffer concentration before KCl addition. In the experiments with myosin or MHC, satisfactory precipitation was not only observed at a highly acid pH, but also at an alkaline pH, near the *pI* value of MHC. On the other hand, albumin, an anionic protein, remains largely soluble above pH 7.0 (Table 2). At constant ionic strength, the solubility of native proteins increases at pH values far from their isoelectric point. That this variable could also be important for the KDS-protein is a new, unexpected observation, because SDS should overcome the native electric charge. An anionic protein was concentrated more effectively at an acid pH, while cationic proteins were also efficiently precipitated at a basic pH. This is an additional advantage of the method which allows fractionation of proteins with different *pI*.

To optimize the method, the temperature and relative concentration of SDS and buffer must be carefully controlled. Indeed, when we analyzed the influence of different SDS concentrations (from 0.05 to 2%) in preliminary experiments at a constant pH of 6.8 and at room temperature (results not shown), MHC recovery was poor because of the increased solubility of KDS-protein at room temperature as well as the large KDS pellets (1–2 mL) in samples at high

SDS and low Tris buffer. At high SDS the Tris-glycine concentration is important: to concentrate proteins at 0°C and 25 mM Tris, 192 mM glycine, pH 8.3, the best SDS concentration is 0.1% w/v. At higher SDS levels, large pellets will form, preventing good protein recovery and concentration (Sandri, M., Rizzi, C., Carraro, U., unpublished data). If no more SDS is added (*i.e.*, the only molecules are those previously bound to protein), the most important variables will be the pH and KCl concentration. Under such conditions, high amounts of Tris-glycine (125 mM Tris, 960 mM glycine, pH 8.3) reduce protein recovery, probably because they increase ionic strength and therefore KDS-protein solubility. Therefore, more effective KDS-protein precipitation is achieved after dilution of the highly concentrated alkaline buffer (compare Fig. 3 A, 8–10 with Fig. 3 B, 1–3). The KCl concentration seems to be less dependent on other variables, *i.e.*, 180 mM KCl effectively precipitates KDS-protein complexes. At a lower KCl concentration the KDS-protein is not adequately precipitated, in agreement with results of Prussak *et al.* [33] who reported that under their conditions (50 mM KCl added to 0.01% SDS, 1 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.9) more than 95% of the myosin and albumin were recovered in the supernatant after the addition of KCl. At concentrations of free SDS higher than 0.5%, a higher Tris-glycine concentration at an alkaline pH results in smaller pellets in which KDS-protein micelles are better concentrated while free dodecyl sulfate remains at least partially in the supernatant (Sandri, M., Rizzi, C., Carraro, U., unpublished data). This opens the possibility of using the method to separate free from bound ligands in labeling studies (also see [26]). Although the results reported here focus primarily on the use of KCl to precipitate SDS-solubilized muscle proteins and, specifically, myosin subunits from EPGE eluates, the consideration that a large proportion of the KDS-precipitate proteins of muscle homogenate are products of genes expressed in all tissues explicitly indicates that the method will effectively precipitate almost all polypeptides.

In conclusion, KCl precipitation of SDS-solubilized proteins is, in our opinion, the most rapid, inexpensive, and effective method to concentrate proteins from highly solutions. While SDS solubility is relatively high (1 g/10 mL, *i.e.* 0.3 M) on addition of KCl it falls almost to zero, resulting in precipitation of KDS-protein micelles. A similar approach was applied to DNA precipitation: Using a detergent similar to SDS, *e.g.* cetyltrimethylammonium bromide, which binds DNA, it was recently demonstrated that it is possible to precipitate nucleic acids much more easily than by standard methods [35]. In biotechnology the recovery of SDS-solubilized biopolymers will be greatly facilitated by KDS precipitation. After precipitation the hundred- or thousand-fold concentrated solutes are easily dissolved in small volumes of any desired medium. We believe that KCl precipitation of SDS-solubilized molecules will find widespread application in the near future, since its major drawback, *i.e.*, protein denaturation, could be overcome in numerous cases by efficient methods of detergent removal and protein renaturation [25, 30–33].

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